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Measurement of cooling and warming rates in vitrification-based plant cryopreservation protocols

Aline S. Teixeira

ICTAN, Instituto de Ciencia y Tecnología de Alimentos y Nutrición, CSIC, José Antonio Novais 10, Madrid, 28040, Spain.

Current address: Centro de Investigación y Desarrollo en Criotecnología de los Alimentos (CIDCA-CONICET), Fac. Cs. Exactas (UNLP), 47 y 116, La Plata (1900), Argentina.

M. Elena González-Benito

Dpto. de Biología Vegetal, E.U.I.T. Agrícola, Universidad Politécnica de Madrid, Ciudad Universitaria, 28040, Madrid, Spain.

Antonio D. Molina-García

ICTAN, Instituto de Ciencia y Tecnología de Alimentos y Nutrición, CSIC, José Antonio Novais 10, Madrid, 28040, Spain.

Correspondence concerning this article should be addressed to A.S. Teixeira or A.D.Molina-García at teixeiraline@hotmail.com or antoniom@ictan.csic.es

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Abstract

Cryopreservation protocols include the use of additives and pre-treatments aimed to reduce the probability of ice nucleation at all temperatures, mainly through micro-viscosity increase. Still, there is a risk of ice formation in the temperature region comprised between the equilibrium freezing ($\mathbf{T}_f$) and the glass transition ($\mathbf{T}_G$) temperatures. Consequently, fast cooling and warming, especially in this region, is a must to avoid ice-derived damage. Vitrification and droplet-vitrification techniques, frequently used cryopreservation protocols based in fast cooling, were studied, alongside with the corresponding warming procedures. A very fast data acquisition system, able to read very low temperatures, down to that of liquid nitrogen, was employed. Cooling rates, measured between -20 and -120 ºC, ranged from ca. 5 ºC s^{-1} to 400 ºC s^{-1}, while warming rates spanned from ca. 2 ºC s^{-1} to 280 ºC s^{-1}, for the different protocols and conditions studied. A wider measuring window (0 ºC to -150 ºC) produced lower rates for all cases. The cooling and warming rates were also related to the survival observed after the different procedures. Those protocols with the faster rates yielded the highest survival percentages.

Introduction

Cryopreservation of living tissues for their functional recovery is widely employed for the multi-purpose preservation of material of medical, veterinary and crop or wild plant species origin. The basis of cryopreservation is the drastic reduction of most chemical and physical processes in cells. While temperature reduction under the physiological fluids freezing point can only slow these processes, freezing reduces dramatically most biological and chemical activities, due to the associated reduction of water activity. Unfortunately, intracellular ice formation has been found to be lethal for most tissues and specimens. Nevertheless, satisfactorily stable storage conditions can be found for most biological systems when storage is carried out in the glassy state, at temperatures well below the freezing point. When the viscosity of liquids increases, their molecular mobility is reduced. This reduction can be so extreme that most processes are virtually stopped.[1]

The transition between liquid and vitreous state is not a first class phase change and, consequently, is not characterized by an enthalpy increment, but by a step in some of the physical properties of the system, such as the heat capacity, specific volume and apparent viscosity. Some authors consider it as a second class phased change,[2] while others consider the glassy state not to be a true thermodynamic state, but a metastable kinetically-controlled phase.[3] Its very slow molecular mobility guarantees the actual stoppage of most processes. Moreover, ice formation, a process requiring the assembly of an initial nucleus of water molecules and its later growth by water molecules incorporation, driven by Brownian movement, is largely arrested in glasses.[4]

The molecular mobility reduction required for vitrification may be achieved by either a temperature drop or by an increase of the solute concentration. The intracellular concentration in biological systems may be effectively increased by dehydration while the glass transition temperature, $\mathbf{T}_G$, is lower the higher the solvent (water) content is. Consequently, vitrification

can be employed for preservation of systems surviving extreme dehydration, such as many seeds (orthodox), even at room temperature.[5] However, most systems would be damaged by the dehydration degree required and a reduction of temperature is additionally required for vitrification. Cooling below the equilibrium freezing temperature, $\mathbf{T}_f$, gives rise to a problem: the temperature gap between $\mathbf{T}_f$ and $\mathbf{T}_G$ must be crossed and ice can be formed in this region. $\mathbf{T}_G$ in many cryopreservation systems based on vitrification has been recorded at approximately -115/-120ºC,[6,7] while crystallization most frequently occurs between 0º and -40ºC.[6,8]

Ice crystal formation takes place always under the equilibrium freezing temperature, but its initial nucleation step is stochastic in nature, depending on the random rearrangement of water molecules to achieve the initial nucleus conformation able to spontaneously grow. That implies that there is an increasing probability of ice being formed the longer the system is at any temperature comprised between $\mathbf{T}_f$ and $\mathbf{T}_G$. For this reason thermal change (cooling and warming) rates are considered critical for the success of cryopreservation. [8-11]

Cryopreservation is customarily applied to animal, plant and microbial specimens. Plant tissues are often preserved after vitrification protocols,[12,13] which imply a previous set of steps designed to increase solute content and reduce water in cells, as well as to promote the plant natural defences towards stress. Cooling by plunging into liquid nitrogen (LN) completes the procedure. The recovery process is nearly symmetric: firstly re-warming is carried out by immersion in a warm water bath, followed by a rehydration and solute unloading stage, before specimen culture. Two methods frequently used in the cryopreservation of plant germplasm are those named directly as vitrification and droplet-vitrification. A difference among them, relevant to temperature change rate, is the mass (of specimen plus cryoprotectant solution and container) to be cooled or warmed.[14,15]

The need for fast temperature change rates in the vitrification and de-vitrification processes is frequently mentioned as being of crucial importance for the success of the cryopreservation process.[8] In spite of this, measurement of actual cooling and warming rates in these protocols has rarely being performed and reported. Actually, most temperature changes rates reported for cryopreservation processes correspond to animal or human origin samples, where the applied protocols are often of different nature. Performing reliable fast thermal measurements in plant cryopreservation systems is a difficult task and therefore the reduced number of reports of cooling/warming rates in these systems, some of them lacking precision in the reported data or a detailed methodology description.[16-19]

In the present work, the rates of cooling and warming in cryopreservation experiments, performed using mint shoot tips and following the vitrification and droplet-vitrification protocols, were determined using a fast temperature measurement system. The viability of apices after cryopreservation was also studied, to ascertain the validity of the protocols followed. While the accepted fact is that plant cryopreservation protocols involving vitrification have an increased success with faster cooling and warming rates, no systematic and comparative study has been published to date. This study presents detailed temperature change rates during the whole cooling and warming steps of several cryopreservation protocols. The reported rates are not only compared amongst these protocols but also related to the viabilities achieved, using the same biological system. Moreover, the equal importance of warming and cooling rates, frequently stressed, is also supported here by experimental data.

Materials and Methods

Plant material pre-culture and shoot tip extraction

Shoot tips were extracted from *in vitro* shoots *of Mentha ×piperita* (genotype "MEN 186" obtained from the IPK Genebank, Gatersleben, Germany). *In vitro* plants were monthly subcultured on medium MS[20] with 3% sucrose, and incubated at constant temperature (25°C) with a photoperiod of 16 h, and an irradiance of 50 µmol m^{-2} s^{-1} from fluorescent tubes. One-node segments were obtained from these shoots, transferred to fresh medium and incubated at alternating temperatures of 25°C (day) and -1°C (dark), always with 16 h photo- and thermoperiod, 50 µmol m^{-2} s^{-1} irradiance, provided by fluorescent tubes.[15] After 3-weeks of culture under these conditions, shoot tips (1-2 mm, covered by 1-2 pairs of leaf primordia) were excised from axillary buds.

Dehydration of shoot tips in the vitrification method

All excised shoot tips were pre-cultured for 24 h on filter paper in liquid MS medium containing 0.3 M sucrose, at 25ºC. Thereafter, the explants were transferred to a Petri dish with 2 mL of loading solution[21] (2 M glycerol + 0.4 M sucrose) over filter paper, for 20 min, at room temperature. Finally, they were osmotically dehydrated in 2 mL PVS2 (plant vitrification solution 2: 30% w/v glycerol, 15% w/v ethylene glycol, 15% w/v DMSO and 0.4 M sucrose in half-strength MS liquid medium[6]) in a Petri dish, on filter paper, for 30 min at 0ºC. All constituents of PVS2 were autoclaved except DMSO, which was filter-sterilised. All solutions stated here or thereafter were prepared in liquid medium MS.

Vitrification

Modifications of the protocols for mint from Volk and Walters (2006)[22] and Uchendu and Reed (2008)[23] were used. After dehydration in PVS2, ten shoot tips were placed in a 1.0 mL cryovial containing 0.5 mL or 1.0 mL PVS2. Then, this container was submerged into LN and, after a short period in these conditions (approx. 1 h), the cryovial was removed and

rewarmed in 40°C water for 2 min, stirring gently. Then shoot tips were washed with 1.2 M sucrose for 20 min at room temperature and subsequently cultured.

Droplet-vitrification

Modifications of the protocol developed by Senula et al. (2007)[15] for mint were studied. After dehydration in PVS2, shoot tips were transferred into droplets of 2 µL PVS2 placed on aluminium foil strips (AFS; 5 droplets per strip, each containing a shoot tip). The strips with the adhered droplets were immersed into LN, either directly (naked) or placed inside cryovials. After a short period in LN conditions (approx. 1 h), cryovials/AFS were retrieved and warmed. Cryovials were plunged into a water bath at 40°C for 3 to 5 s; the AFS were then removed from the cryovial and the apices were incubated in a 1.2 M sucrose solution for 20 min at room temperature. When naked aluminium strips had been immersed in LN, they were directly plunged into the 1.2 M sucrose solution at room temperature and shoot tips incubated for 20 min.

Plant recovery and viability

Shoot tips exposed to the vitrification or droplet-vitrification techniques were cultured on re-growth medium (semi-solid MS medium supplemented with 0.5 mg/L 6-benzylaminopurine and 3% sucrose), incubated in the dark for 24 h and thereafter under 16 h photoperiod, at 25ºC. For control samples (-LN), all steps were carried out except immersion in LN and rewarming.

Survival and re-growth were calculated as percentages over the total number of shoot tips used 4 weeks after culture. Survival was defined including all forms of visible viability (evidence of green structures or callus). Re-growth was defined as the formation of small plantlets. The number of replicates was 3 (control) or 6 (samples) for the vitrification and

droplet-vitrification protocols. Each replicate included 10 shoot tips for the former and 5 for the latter procedure (see above). Data were subjected to arcsin transformation prior to analysis of variance and Duncan's Multiple Range Test (Alpha = 0.05) was used for comparison of means.

Temperature measurement and cooling and warming rates

The specimen temperature change data were acquired using a fast acquisition multichannel temperature measuring system, MW100-UNV-H04 (Yokogawa, Tokio, Japan). Several reading speeds were tested and finally a recording frequency of 20 data per second (measuring interval 50 μs) was selected. Calibration was performed frequently between measurements, using liquid nitrogen and ice for calibration points (-196 and 0ºC, respectively). The cooling and warming rates were defined as the ratio of a predetermined temperature interval, $\mathbf{T}_1$-$\mathbf{T}_2$, over the time elapsed between the reported measurements of $\mathbf{T}_1$ and $\mathbf{T}_2$. The determination of cooling and warming rates was carried out at least in triplicate, during the cooling and warming phases of the described protocols.

Thermocouples

Several types of thermocouples and wire gauges were tested and finally T-type copper-constantan thermocouples were selected for their good performance in the temperature region studied (45 to -150ºC). Thin (diameter 0.5 mm) steel-shielded and mineral-isolated thermocouples (TC Medida y Control, S.A., Madrid, Spain) were chosen, in order to minimize any external electromagnetic influence over measurements. They were placed either on the surface of AFS (commercial household aluminium foil, strips of approx. 20 x 5 mm, 5 mg) or in the centre of cryovials, through a hole in the lid, depending on the cryopreservation technique studied.

Controls were set for empty cryovials and thermocouples without specimens. Additional controls were set for evaluating the effect of heat losses through the thermocouple wire, comparing results with those obtained with additional thermocouple tips inserted in the sample. No significant differences were found that could justify a heat loss correction. Thermocouples were frequently calibrated as a part of the measurement set-up, as described above, and especially after any thermocouple exchange or reconnection.

Experimental set-up and temperature change rate measurement design

A scheme of the experimental set up used for determining temperature change rate is shown in **Figure 1.** The experiment design aimed to reproduce the conditions used in customary cryopreservation, which involves a certain degree of variability due to the operator action and a number of factors not completely controlled or reproducible in the protocol (such as the operational delay in handling the different specimens or the differences in stirring and in contact between specimen and containers).

The sample holders (either a cryovial or an AFS) were handled by means of the attached thermocouple wire, by means of isolated tongs, in order to reduce the heat exchange sources. For cooling rate determination, cryovials/AFS were tempered at 1-4ºC before immersion in LN, and data collection started at 0ºC, and continued till a plateau near -196ºC was reached. For warming, data collection started when the specimens were immersed and thermally equilibrated in LN and continued till room temperature equilibrium. Rates were calculated after adopting an ice risk temperature window (cooling: 0ºC to -150ºC, warming: -150ºC to 0ºC), by dividing the thermal difference between the window extremes by their time difference.

Temperature change rates were determined on specimens being submitted to the vitrification protocol, using a cryovial containing 0.5 or 1 mL of PVS2 solution (containing 10 mint shoot

tips), as well as an empty cryovial for blank comparison. Rates were also measured on specimens treated after the droplet-vitrification protocol, including variations of several parameters. Two experimental conditions were tested (with different masses and specimen distributions): an aluminium foil strip with 5 droplets of 2 μL (containing each one a mint shoot tip), naked or inside a cryovial. Two controls were added: AFS with no shoot tip sample, with and without cryovial.

Results and Discussion

Cooling and warming rates were measured, as described, for vitrification and droplet cryopreservation methods. **Figure 2** shows the temperature versus time data obtained in cooling and warming experiments corresponding to the vitrification protocol. **Figure 2a** shows the temporal evolution during cooling of the sample, consisting of 10 shoot tips in a 1.0 mL cryovial containing 0.5 mL or 1.0 mL PVS2. **Figure 2b** shows data for the same samples during warming. A control performed with an empty cryovial is also shown in both cases. Given the relatively slow rates for this case, the data points obtained at 50 μs intervals form a continuous line on the plot. In spite of the rate visible dependence with the thermal difference with the surrounding fluid, data could not be properly fitted to a function, as many smaller scale phenomena have a variable influence on both cooling and warming processes. These phenomena include changes in heat capacity and conductivity of the containers, fluids and sample components during this wide temperature span considered, as well as possible phase changes (including vitrification) involving heat effects, the heat interchanges derived from mechanical processes when the volume of components is altered, etc. Also the complex convention of the external fluid (and Leidenfrost effect in LN) would generate differences in the instantaneous heat exchange rates.[24, 25]

The cryoprotecting solution content appeared to be a major determinant of the rate, both for cooling and warming: the empty cryovial, with less total heat capacity than those with solution, showed faster rates. Those with samples of 0.5 and 1.0 mL volume did present a very similar behaviour, probably compensating the higher heat capacity of the larger sample and its higher heat conductivity, due to its larger heat exchange surface. The trends observed were quite symmetric for cooling and warming, both for samples and control measurements.

Control observations carried out using several thermocouple wires indicated that any effect of the thermocouple itself on the recorded thermal change rate was negligible. This allowed discarding underestimations of the measured rate resulting from its wire contributions to thermal mass or to heat fluxes through it (data not shown).

Figures 3 and **4** show cooling and warming experiments corresponding to the droplet-vitrification protocol. **Figure 3a** and **b** show temperature temporal evolution determined during cooling by immersion in LN, for different masses and sample distributions (naked AFS or inside a cryovial and with different number of droplets).

Figure 4a and **b** show temperatures obtained during the warming process, carried out as described in the droplet-vitrification protocol, for the same samples as in **Figure 3**. Data obtained were fairly reproducible and, in spite of the additional data scattering due to specimen variability and operator manipulation, their error was much smaller than the differences observed among different experimental conditions. Comparison of these results showed a diverse behaviour in both cooling and warming rates for the different conditions. The presence of drops of PVS2 cryoprotecting solution in this technique induced a reduction of both cooling and warming rates, when compared with the same methodology without the drops.

In those systems using an aluminium foil strip, the small additional mass of this strip (aprox. 3-4 mg) could be compensated by the increased heat diffusion granted by the larger

interchange surface, and it could be observed that both cooling and heating rates of these strips (with the measuring thermocouple attached) were higher than those determined with the thermocouple alone.

The rates in the warming process for this protocol showed larger differences from those of cooling than in the case of the vitrification protocol, due to the particularities of the warming process. Placing the AFS + 5 x 2 μL PVS2 drops sample in the sucrose solution at room temperature had the effect of a reduction of the warming rate, probably due to different interface contact and heat exchange behaviour than that resulting from the immersion in LN. Because of this slower rate, the warming process of this sample has been shown in a different sub-figure than the cooling, faster, one.

In **Figure 4b,** a slope change in the warming process can be observed for the two samples considered, at 0.3-0.4 s. This can be related to the physical transfer from nitrogen to air and then to the warming medium. The slope change is not evident in the data sets of **Figure 4a** probably because it was obscured by the larger duration of the warming process. However, the end of the 3-5 s warming step in the water bath (and exit to room temperature air), for the two samples treated in such a way and shown in **Figure 4a)**, did not give rise to any warming rate irregularity. This may be a result of both these samples being included in a cryovial, whose isolation could mask fast thermal effects. As expected, when comparing the results from both vitrification and droplet-vitrification protocols, it can be seen that the inclusion of specimens in a cryovial considerably reduces both cooling and warming rates.

The data dispersion in the thermal change rates observed comprises both the effect of real experimental errors and that derived from the possible small variations of the cryopreservation procedure. The experimental methodology followed was intended to reproduce the usual operations of cryopreservation practice, rather than to minimize the causes of variability, so that the sources of variation associated to manual manipulations, such

as sample transference between fluids, holding them with tongs and stirring degree, have not been standardized. In the same way, an extreme important factor, as it is the position of the active thermocouple tip within the experimental set-up, especially inside cryovials, has not been fixed. A controlled position in the cryovial centre would have produced more homogeneous data, but it would not have been representative of the real temperature values in positions closer to its wall. All these factors added to an increase in the dispersion of the obtained data.

Figure 5 shows how control specimens, not immersed in liquid nitrogen, presented a 100% survival, proving that the dehydration and chemical treatments applied (including exposure to PVS2) have no toxic effect that could damage apices. Survival and regrowth values were fairly similar for all cases. The highest regeneration percentage was observed (96%) when the droplet-vitrification procedure was employed with direct immersion in LN, the percentage decreasing with the inclusion in a cryovial. The vitrification protocol yielded similar regeneration values for the treatments with 0.5 or 1 mL PVS2 solution inside the cryovial, but much lower (about 43%) than the droplet-vitrification protocol.

General discussion

The observed percentages can be directly related to physical characteristics of specimens and experimental systems employed (containers and cryoprotecting solutions), especially their mass, heat capacity and heat conductivity. For example, those systems with a larger mass will have a higher inertia towards temperature change. The more relevant factor would be the heat amount that the system requires to absorb or to yield for causing a temperature change, i.e. its total heat capacity. The contribution of the different heat capacities of the system components must be accounted for. A higher thermal inertia can be expected when the water content (with

a large specific heat capacity) is high, while the mass of cryovials and aluminium foil would represent a lesser influence, due to its lower heat capacity.

On the other hand, the temperature change is determined not only by the heat amount to be exchanged, but by the rate of this transfer. The different experimental systems employed present many diverse characteristics on this respect. The presence of containers always represents an additional barrier for heat exchange. The position of the specimens inside the cryovial partly determines this behaviour, as depending on the extent of its contact with its walls, heat transfer could be very different. This may explain the complex effect of the presence of vitrification solutions inside the cryovial. On one hand, the thermal mass increases and the total heat amount to be transferred is higher. But this solution could constitute a thermal bridge between the cryovial walls and the specimen, improving heat exchange and so fastening the temperature change process.

The data obtained and presented in **Figures 2-4** show a continuous temperature variation with time. As it can be seen, the slope of the curves (i.e., the instantaneous rate) changes continuously, decreasing as the driving heat gradient declines. In order to provide a single figure rate to enable comparisons, a "temperature window" must be defined. This also produces a parameter more interesting for cryopreservation purposes than the temperature change rate itself: the time of permanence in the ice formation risk area.

The purpose of cryopreservation procedures is to keep samples in the glassy state. Ice crystal formation is considered to be extremely unlikely in vitrified solutions, as ice nucleation and crystal growth are phenomena involving relatively large scale molecular reorganizations. The procedures previous to plunging samples into LN are endeavouring to reduce the likelihood of ice formation in any case (mainly by increasing viscosity and reducing the water available). Nevertheless, there is a definite risk of ice formation during the cooling or warming processes crossing the area below the freezing point and over the glass transition temperature.[26,27]

Ice crystals will never be formed in the temperature regions over $\mathbf{T}_f$ (strictly, over the ice nucleation temperature, $\mathbf{T}_n$) and under $\mathbf{T}_G$. To include these regions in the calculation of rates or related time parameters may be misleading. For example, the time employed in cooling a specimen for $\mathbf{T}_G$ to LN temperature could be relatively large, and the same will happen from $\mathbf{T}_n$ to 0ºC, during the warming process. A good knowledge of a given system to be cryopreserved would allow setting a more adjusted temperature window. For comparison purposes, here we have defined two windows: from 0 to -150ºC and from -20 to -120ºC. The first one, spanning from pure water equilibrium freezing point (the higher temperature where ice can exist, in any aqueous solution) to -150ºC, well below any expected glass transition in a living system, is largely overestimated over the real ice formation risk zone for systems in cryopreservation, as its $\mathbf{T}_f$ will be always much lower than 0ºC, due its high solute concentration and low water content. The variability in the glass transition temperature is high, being very sensitive to the content in water and other small size molecules,[28,29] but, for plant viable germplasm, is usually well over -150ºC. Moreover, the thermal gradient ($\Delta\mathbf{T}$, the difference between sample and surrounding media temperatures) is similar in the initial and final windows extremes, for both cooling and warming (using a 40ºC bath). For example, for the 0 to -150ºC window, $\Delta\mathbf{T}$ would evolve from 196ºC at the initial point, to 46ºC at the window end-point, for cooling, and from an initial value of 190ºC to a final one of 40ºC, for warming. This results in the generation of similar cooling and warming rates (in absolute value), as other contributing phenomena will have a more reduced weight, due to the high speed of the process (such as changes in convention layer or geometrical alterations in sample and/or containers).

The narrower temperature window, from -20 to -120ºC, was fixed taking into consideration the freezing point observed for mint shoot tips in previous stages of the droplet cryopreservation protocol.[7] The specimens at the final stage of the protocol, actually those

plunged into LN, are reported to present no freezing event in calorimetric conditions (at a cooling rate of 10ºC min^{-1}), but we have considered that a temperature of -20ºC would be a safe limit for ice formation, considering also the usual reduction of the nucleation temperature with respect to the equilibrium freezing one. The -120ºC limit follows the reported $\mathbf{T}_G$ for this system on -119ºC.[7]

Table 1 and **2** show the calculated cooling and warming rates and permanence times obtained for these two windows for experiments of **Figures 2** to **4**. In all cases, the narrower (-20 to -120ºC) window gave higher rates. This was due to the larger temperature gradient, as the region closer to the thermal process end point (always showing slower rates) was left out of the calculation. The more adjusted the window is to the real ice formation probabilities, the more relevant the rate and times calculated would be. Anyhow, it must be noted that the probability of ice formation is not constant for the whole window. Its dependence with temperature is complex and difficult to study, but it increases as temperature descends from the equilibrium freezing point, being counterweighted by the reduction on molecular mobility, also driven by lower temperatures. Near $\mathbf{T}_G$, for time spans of the order of seconds, this probability is most likely very small. A region of about 20ºC over the $\mathbf{T}_G$ could be considered also safe for these purposes (for short permanence, not for long term storage), as it has been reported that its high viscosity grants that any relatively large scale change (such as ice formation) would take place very slowly or not happen at all.[30]

The main difference between the cryopreservation methods used in this work is the amount of PVS2 in contact with the specimen. An advantage of the droplet-vitrification protocol is the possibility of achieving very high cooling/warming rates, due to the very small volume of cryoprotective medium in which the explants are placed. The significance of the highest rates reported here must be taken with prudence. The parameter directly measured is (apart from temperature), the time span between two temperature readings. In the faster processes, the

fraction of this time span that could correspond to manipulations of the operator when transferring samples can be non-negligible, and when rates are calculated by division of these two quantities (temperature interval and time lapse), very high differences can be so generated among measurements, perhaps not representing reality.

Resorting to fast temperature changes in cryopreservation has been broadly studied and employed to reduce ice crystal size. Luyet and co-workers (1965)[31] pioneered the use of rapid (non-equilibrium) cooling as a means of restricting the size of ice crystals during cryopreservation of hydrated samples. For example, cooling rates in excess of 100°C s^{-1} are reported to facilitate the preservation of microorganisms and a variety of animal plant and insect origin material ([8] and references therein). In spite of the crucial role of thermal change rate on cryopreservation, few studies include detailed measurements of the evolution of temperature in these conditions.[8,32-35] High viabilities in recovered tissues have been reported at elevated cooling rates (40-400ºC s^{-1}).[8,17,36] The rates reported by these workers with sweet potato shoot tips, in spite of the different material, show good agreement (6ºC s^{-1}) with our results for cooling inside a cryovial, while for cooling on exposed AFS, a lower value (130ºC s^{-1}) was reported, when compared to our 350-500ºC s^{-1} data. The rates reported by Hirai (2011)[19] in a different system were much lower (1.6 ºC min^{-1} for the vitrification procedure and 91.4 ºC min^{-1}, for droplet vitrification). Nevertheless, a similar viability behaviour was observed, with a significant higher recovery rate at larger cooling rates.

The need to use a high rate to avoid ice formation during cooling (and warming) has especial importance when the specimens' water content is high. However, the rates required to avoid ice formation cannot be practically reached when water content is very high, for a sample of the size required for plant germplasm successful manipulation and cultivation. It is often necessary to act on the specimen to reduce the probabilities of ice formation, generally decreasing water content, with the multiple effect of reducing the ice formation risk window

(both by lowering $\mathbf{T}_f$ and, at the same time, increasing $\mathbf{T}_G$), while the increase in intracellular viscosity limits the ice formation probability, even within this window. So, partial dehydration reduces the requirements of very fast cooling.

Relatively slow temperature variations, as those reported in this study for procedures using cryovials (1-7ºC s^{-1}) would be inadequate for application to high water content systems. However, the higher rates (100-500ºC s^{-1}) obtained in some methods using aluminium foil strips, would be appropriated for these less dehydrated specimens. The rate differences associated to the shoot tip water content, would be, however, small in cryovials-based methods (differences between 2 and 4ºC s^{-1}), being the limiting effect the thermal diffusion and the very cryovial mass.

Conclusion

Mint shoot tip survival results suggest that the observed physiological response is dependent upon the cryopreservation method and the cooling and warming rates associated. This study demonstrates that faster thermal rates give rise to higher survival and recovery percentages.

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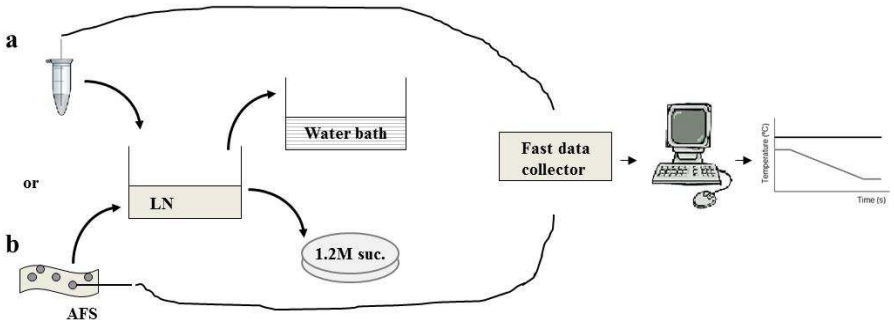

Figure 1. Graphical representation for the procedure followed for thermal change rate measurement, following standard cryopreservation protocols. Vitrification (**a**), where shoot tips were contained in a cryovial, or droplet-vitrification (**b)**, where shoot tips were placed on an aluminium foil strip (AFS), inside a cryopreservation solution droplet. Liquid nitrogen (**LN**).

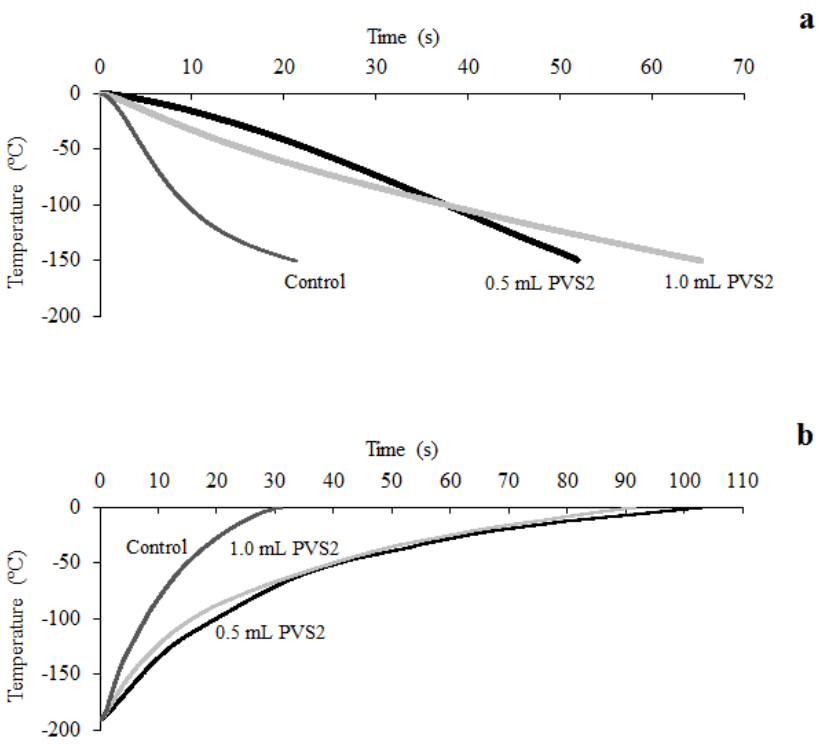

Figure 2. Cooling (a) and warming (b) curve experimentally determined for shoot tips treated following the vitrification protocol. Data are the average of at least three independent determinations. Control data were obtained with the thermocouple tip inserted in an empty cryovial. The lines shown represent the individual data points measured. Plant vitrification solution 2 (PVS2).

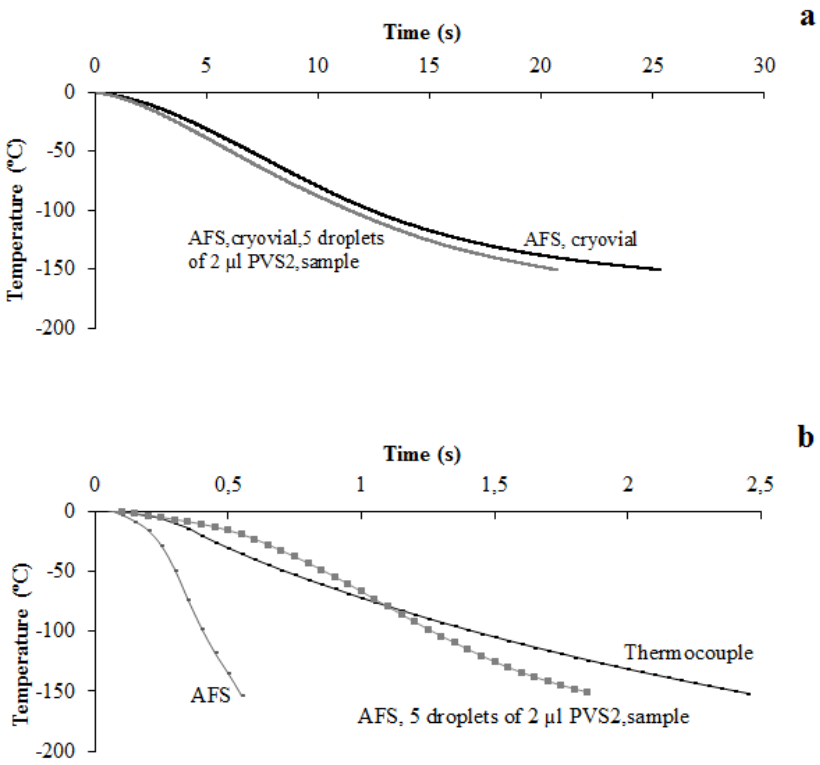

Figure 3. Cooling temperature evolution obtained following the droplet-vitrification protocol: aluminium foil strip (AFS) (**a**) inside cryovial and naked AFS (**b**). Data are the average of at least three repeats. Plant vitrification solution 2 (PVS2).

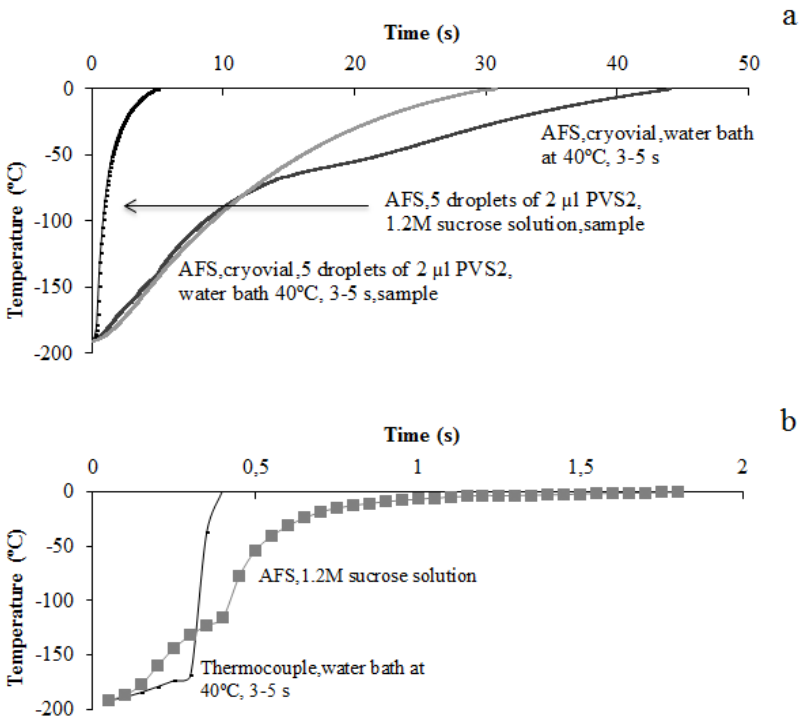

Figure 4. Temperature evolution during warming after the droplet-vitrification protocol: **a** and **b** show different time scales, for the better appreciation of faster changes. Data are the average of at least three repeats. Aluminium foil strip (AFS); plant vitrification solution 2 (PVS2).

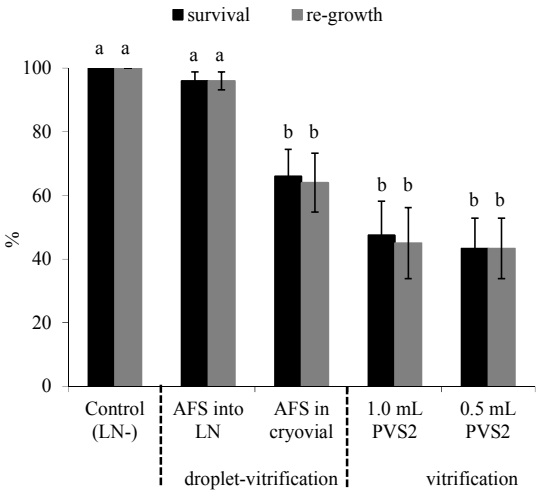

Figure 5. Effect of cryopreservation protocols on the survival and re-growth of cryopreserved *Mentha ×piperita*. Shoot tips had been submitted to droplet-vitrification (AFS into LN or AFS in cryovial) or vitrification (0.5 or 1.0 mL PVS2) cryopreservation protocols. Control specimens were pretreated and cultivated, excluding the cooling and warming steps. Aluminium foil strip (AFS); liquid nitrogen (LN); plant vitrification solution 2 (PVS2). Means of survival or re-growth with the same letter were not significantly different, after the Duncan's Multiple Range Test at Alpha = 0.05. Bars: standard error.

Table 1. Cooling and warming rates obtained in mint shoot-tips cryopreservation following the vitrification protocols.

Method	Window (ºC)	Cooling		Warming	
		Time[1] (s)	Rate[1] (ºC s^{-1})	Time[1] (s)	Rate[1] (ºC s^{-1})
Control: cryovial → LN → bath 40ºC	0/-150	21.0 ± 0.2	7.14 ± 0.01	27.8 ± 0.4	5.4 ± 0.6
	-20/-120	10.0 ± 0.1	10.0 ± 0.1	16.3 ± 0.3	6.1 ± 0.7
Cryovial with 0.5 mL PVS2[2] → LN → bath 40ºC	0/-150	52 ± 1	2.88 ± 0.02	95 ± 1	1.58 ± 0.01
	-20/-120	31.43 ± 0.5	3.18 ± 0.04	55.1 ± 0.9	1.81 ± 0.02
Cryovial with 1 mL PVS2[2] → LN → bath 40ºC	0/-150	65 ± 1	2.31 ± 0.02	86 ± 1	1.74 ± 0.01
	-20/-120	41.9 ± 0.9	2.39 ± 0.03	54.0 ± 0.8	1.85 ± 0.02

[1]Average values and standard deviation for at least three repeats. [2]Experiment performed with shoot tips in the cryovial. **Window**: temperature interval selected for time and rate calculation. **Time**: that spanning between the recording of the window initial and final temperatures (either during cooling or warming). **Rate**: calculated as the ratio between the window and the corresponding time.

Table 2. Cooling and warming rates obtained in mint shoot-tips cryopreservation following the droplet-vitrification protocols.

Method	Window (ºC)	Cooling		Warming	
		Time[1] (s)	Rate[1] (ºC s^{-1})	Time[1] (s)	Rate[1] (ºC s^{-1})
Thermocouple → LN → bath 40ºC (3-5 s)	0/-150	2.32 ± 0.05	65 ± 1	0.09 ± 0.03	1700 ± 500
	-20/-120	1.38 ± 0.05	72.5 ± 2	0.05 ± 0.03	1887 ± 700
AFS → LN → 1.2M sucrose room temperature	0/-150	0.43 ± 0.03	350 ± 25	1.59 ± 0.05	94 ± 2
	-20/-120	0.20 ± 0.03	500 ± 70	0.32 ± 0.03	310 ± 30
AFS inside cryovial → LN → bath 40ºC (3-5 s)	0/-150	25.10±0.30	6.00 ± 0.05	40.0 ± 0.4	3.75 ± 0.03
	-20/-120	11.80 ± 0.10	8.5 ± 0.1	26.5 ± 0.4	3.77 ± 0.04
AFS with 5 x 2 µl PVS2 droplets[2] → LN → 1.2 M sucrose room temperature	0/-150	1.35± 0.05	110 ± 3	4.58 ± 0.08	32.8 ± 0.4
	-20/-120	0.94 ± 0.04	106 ± 3	2.03 ± 0.04	49 ± 1
AFS with 5 x 2 µl PVS2 droplets[2] in a cryovial → LN → bath 40ºC (3-5 s)	0/-150	20.6 ± 0.3	7.28±0.06	26.4 ± 0.5	5.68 ± 0.03
	-20/-120	11.00 ± 0.02	9.1 ± 0.1	15.4 ± 0.2	6.49 ± 0.08

[1]Average values and standard deviation for at least three repeats. [2]Experiment performed with shoot tips in the cryovial. **Window**: temperature interval selected for time and rate calculation. **Time**: that spanning between the recording of the window initial and final temperatures (either during cooling or warming). **Rate**: calculated as the ratio between the window and the corresponding time.